Is docosahexaenoic acid, an n−3 long-chain polyunsaturated fatty acid, required for development of normal brain function? An overview of evidence from cognitive and behavioral tests in humans and animals[1–3]

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ABSTRACT

This review is part of a series intended for nonspecialists that will summarize evidence relevant to the question of whether causal relations exist between micronutrient deficiencies and brain function. Here, we focus on experiments that used cognitive or behavioral tests as outcome measures in experimental designs that were known to or were likely to result in altered brain concentrations of the n−3 fatty acid docosahexaenoic acid (DHA) during the perinatal period of "brain growth spurt." Experimental designs reviewed include observational breastfeeding studies and randomized controlled trials in humans and studies in rodents and nonhuman primates. This review is based on a large number of expert reviews and commentaries and on some 50 recent studies in humans and animals that have not yet been included in published reviews. Expert opinion regarding the strengths and weaknesses of the major experimental systems and uncertainties associated with interpreting results is summarized. On the basis of our reading of this literature, we conclude that evidence from several types of studies, particularly studies in animals, suggests that, within the context of specific experimental designs, changes in brain concentrations of DHA are positively associated with changes in cognitive or behavioral performance. Additional experimental information required to conclude that a causal association exists is discussed, as are uncertainties associated with applying results from specific experimental designs to the question of whether infant formula should be supplemented with DHA. *Am J Clin Nutr* 2005;82:281–95.

KEY WORDS Long-chain polyunsaturated fatty acids, LCPUFAs, docosahexaenoic acid, DHA, linolenic acid, cognition, learning, memory, perinatal period, breastfeeding, formula feeding, essential fatty acids, n−3 fatty acids, brain, neurology, infants, childhood, rodent studies, nonhuman primate studies

INTRODUCTION

During the last trimester of fetal life and the first 2 y of childhood, the brain undergoes a period of rapid growth termed the "brain growth spurt" (1). A large and dispersed literature suggests that an inadequate supply of any of a number of essential micronutrients during this period, as well as throughout life, can compromise brain function (2–9). In addition to the enormous literature examining the effects of individual micronutrients, some studies have examined the effects of multivitamin and mineral supplementation on cognitive function (10, 11).

If a causal relation between micronutrient deficiencies and suboptimal brain function exists, it has major public health implications. Large segments of the world population (including the United States), particularly the poor, are known to be undernourished in a number of micronutrients (12–19). A major effort to address micronutrient undernutrition, as an adjunct to the various programs underway to improve dietary habits, particularly among the poor, will be well justified. One of us has discussed such an approach as a relatively inexpensive and efficacious adjunct to current public health programs (20, 21). This review is the first of a series intended to provide the nonspecialist with critical summaries of the available experimental evidence pertinent to a discussion of this important public health question (20, 21).

The goal of this review is to provide an overview of human and animal in vivo experiments that link the availability during development of the long-chain polyunsaturated fatty acids (LCPUFAs), particularly the n−3 LCPUFA docosahexaenoic acid (DHA, 22:6n−3), to performance on cognitive or behavioral tests. Primary resources were recent research reports not yet reviewed, key earlier studies, and a large number of expert reviews and commentaries. We searched the literature by using a combination of techniques including key word and author

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searches of the National Library of Medicine's PUBMED database and the Science Citation Index Cited References database. We also surveyed citations included in recent research and review articles. Abstracts were not included.

General background

LCPUFAs, particularly DHA and arachidonic acid (AA), are highly concentrated in cell membranes of the retina and brain (22–26), and they accumulate rapidly during the brain growth spurt (27, 28). Whereas AA is known to be involved in cell signaling pathways and to be a precursor of eicosanoids that are important for a number of cellular processes (29–31), specific functional roles for DHA in the brain are less well elucidated. Examples of research and reviews of this important topic are available in many publications (31–59).

Reduced visual acuity has consistently been observed in primate and rodent offspring subjected to dietary conditions during gestation that result in significant reductions in retinal concentrations of DHA (60–65). Some formula-comparison studies in humans, particularly in very-low-birth-weight (VLBW) and preterm infants, suggest that greater visual acuity in infancy is associated with increased intake of LCPUFAs (66–68), and these results are supported by several meta-analyses (69–71). The conclusions of systematic reviews differ somewhat, however, as to the overall strength of evidence for a positive effect of LCPUFA-supplemented infant formula on visual acuity (30, 69–74). Numerous publications provide additional review (4, 30, 57, 75–80). Effects of DHA availability during development on visual acuity are not a focus of this review but are discussed as a potential confounder of results in some cognitive and behavioral tests.

DHA is not widely distributed in the diet but is present in some foods, particularly fatty fish (30, 81–84). DHA can also be synthesized in the body from the essential fatty acid (EFA) α-linolenic acid (ALA). Although infants are able to convert ALA to DHA (85–88), DHA is known to be supplied directly to the fetus through the placenta and to the postnatal infant via breast milk (89–93). Uncertainty as to whether endogenous synthesis by the infant is sufficient to supply enough DHA during the postnatal phase of perinatal growth has stimulated a great deal of interest in whether infant formula should be supplemented with LCPUFAs. This general issue has been discussed in many reviews and commentaries (4, 30, 54, 79, 94–100). We do not take up this issue directly in this review, but we do comment on the relevance to this important question of the results from the experimental systems we reviewed.

Basic experimental designs

Three types of protocols were most commonly used in the studies reviewed. Each of these protocols differently manipulates the dietary supply of EFAs or LCPUFAs during development, and all result, or are likely to result, in different brain concentrations of DHA in comparison groups, which makes them relevant to a discussion of whether DHA availability in the brain is linked to cognitive or behavioral function.

Most observational breastfeeding studies in humans compare children who were either breastfed or formula-fed or those who were breastfed for different periods of time. Most randomized controlled trials (RCTs) in humans compare groups fed infant formulas that are either unsupplemented or supplemented with LCPUFAs. Most studies in animals compare offspring whose supply of ALA, the precursor of DHA, has been severely limited for different periods of time.

In most rodent ALA-restriction studies, brain concentrations of DHA are dramatically reduced in test animals, by as much as 85% (101), and decreases of 75% in nonhuman primates have been reported (64). It is important, however, that some studies in rodents also used dietary conditions that resulted in smaller decreases in brain DHA (40, 102–105). Human autopsy studies reported significant differences of ≈11% to 40% in DHA concentrations in brain gray matter between breastfed and unsupplemented formula-fed infants (106–110); two further references provide review (4, 111). Direct autopsy evidence that compares brain DHA concentrations in human infants fed unsupplemented and LCPUFA-supplemented formulas is not available. However, a recent autopsy study in nonhuman primates reported ≈30% lower concentrations of DHA in the visual cortex of preterm infants fed unsupplemented formula than in those fed LCPUFA-supplemented formula (112). In humans, significant differences in plasma concentrations of DHA between unsupplemented and supplemented formula comparison groups are well documented (113, 114).

It is important to note that, in all of these common experimental designs that affect DHA status, DHA is not the only variable. Even in RCTs, formulas typically are supplemented with other LCPUFAs, particularly AA, in addition to DHA. Thus, to different degrees, these designs have an inherent lack of specificity that is important to take into account in evaluating the significance of results relative specifically to DHA.

Performance tests

Many different kinds of tests have been used in human and animal studies. In this review, we focus primarily on tests aimed at assessing cognition or related mental functions, but a few tests assessing other correlates of functional development of the nervous system, such as neuromotor activity (115, 116) and sleep patterns (117), are also included. Several reviews address this important topic (4, 48, 57, 118–127). Tests in human studies include standardized global tests that screen broadly for cognitive-related functions, such as the Bayley Scales of Infant Development (128) and the Kaufman Assessment Battery for Children (129), and tests that target more specific functions, such as the development of language and communication skills [eg, the MacArthur Communicative Development Inventory (130)], visual recognition [eg, the Fagan Test of Infant Intelligence (131)], or problem solving (132, 133). As previously reviewed (57, 75, 118), performance tests in nonhuman primate studies have included assessments of behavior patterns (134, 135) and some tests that have also been used in human studies, most notably "look duration" tests of visual attention (136). Commonly used in rodent studies are tests that measure aspects of spatial learning such as the radial maze (137, 138) and Morris water maze (139–141), the elevated plus maze (a test that measures anxiety; 142, 143), and tests that measure stimulus-response type learning, such as brightness discrimination tests (144, 145). Several reviews provide further discussion of performance tests used in rodent studies (119, 144, 146, 147).

The fact that different tests measure different aspects of cognition or behavior must be taken into account in the interpretation and comparison of results across different tests. For example, in humans, it has been suggested that different results might be

expected in global tests (such as the Bayley Scales of Infant Development) and in tests targeted to more specific neural domains (such as look duration tests), which may not equally measure specific neural functions that may be affected by LCPUFA availability (57, 124). In rodent tests, it has been suggested that a version of the Morris water maze that measures working memory may be more sensitive in detecting performance deficits in n−3–restricted animals than is the more commonly used "place" version of the test (148). Evaluation of results in performance tests must also consider potential confounders. For example, most of the tests listed above rely on vision or motor activity, and thus possible effects of DHA on visual or motor development could theoretically influence the outcome.

HUMAN STUDIES

Breastfeeding studies

Information provided by observational breastfeeding studies, although relevant to the question of causality, is limited. As has frequently been noted (4, 80, 91, 126, 127, 149–153), the decision to breastfeed is associated with a number of factors that potentially confound positive results in these studies, such as socioeconomic status, home environment, and maternal intelligence quotient (IQ). Moreover, experimental designs involving breastfeeding are inherently limited in their ability to identify which among a number of potentially active ingredients in breast milk might be responsible for enhancement effects (4, 91, 127, 154, 155).

Five systematic reviews published since 1999 critically evaluated partially overlapping subsets of breastfeeding studies spanning >20 y (4, 152, 153, 156, 157). Most of the studies included in these reviews compared the performance of children who were breastfed or formula-fed. Before adjustment for covariables, most of these studies reported higher scores on performance tests for children who were breastfed. Reviewers were in agreement that a significant number of potentially confounding variables complicated the analysis and interpretation of these results. However, they agreed less as to whether there was some residual enhancement in test performance after adjustment for covariables. For example, one can compare the conclusions of the studies by Anderson et al (156) and Drane and Logemann (157) and of several critical commentaries (149, 150, 158).

Areas that reviewers suggested for future research included improvement in the overall quality of studies; comprehensive confounder analysis (153, 157); examination of longer durations of breastfeeding, with duration of breastfeeding as a dose-response variable (157); and more studies comparing breast milk–fed and formula-fed premature infants (152).

Breastfeeding studies published in the past 5 y (since 1999), along with formula-feeding studies, are listed in **Table 1**. The table includes an indication of the type of experimental design, the performance tests used, and conclusions of the investigators. Reviews that have cited these studies are also indicated in the table. The range of experimental approaches is greater in these newer studies than in those reviewed earlier, which is consistent with suggestions of earlier reviewers. Thus, whereas several of these studies compared breastfed and formula-fed children (160–167), 12 studies compared the performance of children who were breastfed for different periods of time (159, 168–178).

The great majority of these studies reinforce conclusions of earlier work. Most studies that compared breastfed and formula-fed groups and those that examined groups that were breastfed for different periods of time observed a weak association between scores on performance tests and longer periods of breastfeeding, even after adjustment for at least some potential confounders. As also indicated in Table 1, positive results were reported across a wide range of different performance tests.

It is important to note, however, that these studies do not adjust for all possible confounders. As an example, one of the strongest potential confounders logically is—and has been shown experimentally to be—maternal IQ. As discussed previously (150), in the 1999 Anderson et al (156) meta-analysis of earlier studies, only 6 of the 20 breastfeeding studies cited included maternal IQ as a potential confounder. Among the more recent observational breastfeeding studies listed in Table 1, only 8 included maternal IQ as a potential confounder (159, 160, 169, 174, 177–180). Among those 8 studies, only 2 (159, 177) reported that better test performance by breastfed youngsters remained significantly different after adjustment for multiple confounders. However, a commentary on 1 of these 2 positive studies, that of Rao et al (177), suggested that additional confounders still might account for the residual positive results (149). Specifically, the commentators suggested the inclusion of a verbal measure of maternal IQ (ie, the Peabody Picture Vocabulary Test) and of a measure of parenting quality (such as the Home Observation Measurement of Environment). These measures were significant confounders in a study reported by the commentators (160) that lost statistical significance with adjustment (Table 1). It may be of interest that only one other study (178) among those discussed here used a verbal measure of maternal IQ [all other studies used the Raven's Progressive Matrices (203, 204)]. That study also reported, as discussed above, that adjustment almost completely removed significance (178).

Randomized controlled trials

Studies comparing children fed differently supplemented formulas

Randomized controlled trials (RCTs) offer much greater opportunity than do observational studies for the control of experimental variables, including the quantity and composition of LCPUFA supplements. In addition, this design affords the opportunity to avoid many of the potential confounders that complicate the interpretation of observational breastfeeding studies. RCTs, however, are also subject to confounding and to other sources of uncertainty that can complicate the comparison of results in different trials, such as the use of different formulas or oils or the presence of other sources of variability in the DHA status of infant groups. A general review is available in numerous publications (4, 48, 54, 77, 122, 151, 154, 181, 182).

Results of RCTs have been discussed by many investigators and reviewers. We focused on several systematic reviews and meta-analyses involving term infants (69, 71, 74, 122), preterm infants (70, 73), or both (4, 72, 78–80, 182, 183). Although some of these reviews focused only on visual acuity, the most commonly measured outcome in human RCTs, most reviews also critically examined cognitive or behavioral outcomes (4, 72–74, 78–80, 182, 183). Conclusions varied somewhat among these reviewers as to the strength of the evidence for effects of

TABLE 1
Human studies (1999–2004) that compared effects of breastfeeding or long-chain polyunsaturated fatty acid (LCPUFA)–supplemented or unsupplemented formula feeding on cognitive or behavioral function[1]

Reference	Protocol	Tests[2]	Investigator conclusions	Reviews
Agostoni et al, 2001 (168)	BF duration, 3–12 mo [44]	A (1 y)	Positive trend, adjusted (PDI, $P = 0.07$)	150
Angelsen et al, 2001 (169)	BF duration, <3 to ≥6 mo [345]	A (13 mo); B, C (5 y)	Positive, partial adjusted [A (MDI), B][3]	127, 150
Auestad et al, 2001 (162)	RCT [239]; BFR [165]	R (2, 4, 6, 12 mo); O (6, 9 mo); A (6, 12 mo); EE (9, 14 mo)	Negative	52, 69, 80
Auestad et al, 2003 (163)[4]	RCT [107]; BFR [50]	Z, G, AA, R (39 mo)	Negative, adjusted	
Bakker et al, 2003 (179); Ghys et al, 2002 (180)	Blood LCPUFA concentrations (umbilical at birth and at 4 or 7 y)	M (4 y) [128]; L (7 y) [306]	Negative, adjusted	
Birch et al, 2000 (189)[5]	RCT [56]	A (18 mo)	Positive	49, 72, 78–80, 119, 124, 181–183
Birch et al, 2002 (190)	RCT [65]	BB (17, 26, 39, 52 wk); VEP (6, 17, 26, 52 wk)	Positive (17 wk) (BB); positive (17, 26, 52 wk) (VEP)	69
Bouwstra et al, 2003 (170)	BF duration, <6 wk to 12 mo [147][6]	D (3 mo)	Positive, adjusted	
Bouwstra et al, 2003 (164)	RCT [250]; BFR [147][6]	D (3 mo)	Positive, adjusted	
Cheruku et al, 2002 (186)	Maternal blood LCPUFA concentrations [17]	GG (1,2 d)	Positive	
Colombo et al, 2004 (185)	MS [70]	FF (4, 6, 8, 12, 18 mo)	Positive	
Fewtrell et al, 2002 (167)	RCT [195 PT]; BFR [88]	A (18 mo); U (9 mo)	Negative (RCT); positive; adjusted (BFR)	73
Fewtrell et al, 2004 (184)	RCT [238 PT]	A (18 mo)	Positive (boys)	
Gomez-Sanchiz et al, 2003 (159)	BF duration, ≤4 mo or >4 mo [249][7]	A (18 mo)	Positive, adjusted (MDI)	
Helland et al, 2001 (191) and 2003 (192)	MS [341]	O (6, 9 mo); L (4 y) [84]	Positive at 4 y[8]	52
Hoffman et al, 2003 (193)	RCT [61]	BB (4, 6, 9, 12 mo); VEP (4, 6, 12 mo)	Negative (BB); positive (12 mo) (VEP)	69
Horwood et al, 2001 (176)	BF duration, 0 to >8 mo [280 VLBW]	H (7–8 y)	Positive, adjusted	150
Innis et al, 2001 (188)	Blood LCPUFA concentrations [83]	A (6, 12 mo); O (6, 9 mo); P, Q (9 mo); R (2, 4, 6, 12 mo)	Positive, adjusted (P, R)	52
Jacobson et al, 1999 (160)	BF (mean 6 mo) [323] versus FF [280]	N, G (4 y); H, S, T (11 y)	Negative, adjusted[9]	
Lucas et al, 1999 (165)	RCT [309]; BFR [138]	A (18 mo); U (9 mo)	Negative, adjusted	49, 69, 72, 78–80, 119, 124, 150, 181–183, 194
Makrides et al, 2000 (166)[10]	RCT [68]; BFR [46]	A (1, 2 y); VEP (16, 34 wk)	Negative (RCT); positive, adjusted (BFR) (A, 2 y; VEP, 34 wk)	4, 49, 69, 72, 78–80, 124, 181–183
Makrides et al, 2000 (195)[11]	RCT [58]; BFR [85]	VEP (16, 34 wk)	Negative	4, 78, 181
Morley et al, 2004 (161)	BF [175] versus FF [147][12]	A (18 mo); U (9 mo)	Positive, adjusted (A)	
Mortensen et al, 2002 (171)	BF duration, ≤1 to >9 mo [3253][13]	E (mean 27.2 y); F (mean 18.7 y)	Positive, adjusted	
O'Connor et al, 2001 (196)	RCT [470 PT]; BFR [43][14]	O (6, 9 mo); A (12 mo); EE (14 mo); R (2, 4, 6 mo); VEP (6 mo)	Positive [VEP, EE, O (6 mo), A (PDI)][15]	72, 73, 80, 182, 194
Oddy et al, 2003 (172)	BF duration, 0 to >6 mo [2393][16]	G (6 y) [1450]; H (8 y) [1375]	Positive, adjusted (G)	
Quinn et al, 2001 (173)	BF duration, 0 to 6 mo [3880]	G (5 y)	Positive, adjusted	
Rao et al, 2002 (177)	BF duration, SGA [220]; AGA [299]	A (13 mo); B (5 y)	Positive, adjusted (B)	
Ribas-Fito et al, 2003 (174)	BF duration, <2 wk to >16 wk [92]	A, I (13 mo)	Positive, adjusted[17]	

(*continued*)

TABLE 1 (*continued*)

Reference	Protocol	Tests[2]	Investigator conclusions	Reviews
Richards et al, 2002 (175)	BF duration, 0 to >7 mo [1739][18]	J (15 y); HH (26 y); K (53 y)	Positive, adjusted (J)[19]	
Smith et al, 2003 (178)	BF/HMF duration, <1 wk to >6 mo [439 VLBW]	L, G, V, W, X (6–8 y)	Positive, adjusted (X, L)[20]	
Voigt et al, 2002 (187)[11]	Blood LCPUFA concentrations [44]	A, DD (mean 12.3 mo)	Some suggestive correlations	
Williams et al, 2001 (197)	BF duration, 0 to ≥4 mo; MD [435]	BB (3.5 y)	Positive, adjusted	

[1] *n* is given as numbers in brackets. AGA, appropriate for gestational age; ALA, α-linolenic acid; BF, breastfed; BFR, breastfed reference group included in an RCT; FF, formula fed; HMF, human milk fed; IQ, intelligence quotient; MD, maternal diet comparison; MDI, mental development index; MS, maternal LCPUFA supplementation; PDI, psychomotor development index; PT, preterm infants; RCT, randomized controlled trial; SGA, small for gestational age; VEP, visual evoked potential; VLBW, very low birth weight.

[2] A, Bayley Mental and Psychomotor Development Index; B, Wechsler Preschool & Primary Scales of Intelligence (WPPSI-R); C, Peabody Developmental Motor Scales; D, general movements; E, Wechsler Adult Intelligence Scale (WAIS); F, Børge Priens Prøve (BPP); G, Peabody Picture Vocabulary Test (PPVT-R); H, Wechsler Intelligence Test for Children–Revised (WISC-R); I, Griffiths Scales of Infant Development; J, Group Ability Test AH4 (verbal and nonverbal intelligence), Watts-Vernon Reading Test, and mathematics test; K, National Adult Reading Test (NART); L, Kaufman Assessment Battery for Children (K-ABC); M, Groningen Developmental Scale (adaptation of the K-ABC); N, McCarthy Scales of Children's Mental Abilities; O, Fagan Test of Infant Intelligence (novelty preference); P, conditioned head-turn procedure (speech perception); Q, object search task; R, Teller Acuity Card Procedure (visual acuity); S, Wide Range Achievement Test–Revised (spelling and arithmetic subtests); T, Woodcock Reading Mastery Test-Revised (word and passage comprehension subtests); U, Knobloch, Pasamanick, and Sherrard's Developmental Screening Inventory; V, Clinical Evaluation of Language Fundamentals–Third Edition; W, California Children's Verbal Learning Test; X, Wide Range Assessment of Visual Motor Abilities (WRAVMA); Z, Stanford-Binet Intelligence Scale Form L-M; AA, Beery Visual-Motor Index Test; BB, Preferential looking test for stereoacuity; CC, Clinical Adaptive Test/Clinical Linguistic & Auditory Milestone Scale (CAT/CLAMS); DD, Gross Motor Scale of the Revised Gesell Developmental Inventory; EE, MacArthur Communicative Development Inventories; FF, look duration tests aimed at measuring the development of attention; GG, sleep patterns; HH, highest education or training level.

[3] Confounders evaluated individually, but not combined in the "adjusted scores." Total population was of relatively high socioeconomic status.

[4] Follow-up of children participating in a 1997/1998 study (198, 199) in which children fed the docosahexaenoic-acid-only–supplemented formula scored lower than did other groups on some tests.

[5] Follow-up of an earlier study (200).

[6] Same infants as in Bouwstra et al (164, 170).

[7] IQs of parents evaluated for 164 infants.

[8] Mental Processing Composite of the K-ABC.

[9] Significantly higher IQ scores at both ages after adjusting for social class and maternal education, but differences were no longer significant after adjustment for maternal IQ and parenting skills.

[10] A subsequent study (201) combined data from this and another study (166, 195) to examine a number of perinatal and nutritional variables in relation to the VEP results.

[11] Comparison of formulas supplemented with different concentrations of ALA.

[12] The breastfed group was SGA; the formula-fed groups were term infants. The study also included a group fed a protein and vitamin–enriched formula.

[13] Duration of breastfeeding was determined by interview with mothers; 2 independent groups were assessed: 2280 men and 973 men and women.

[14] Results for a human milk–fed group were not included in the statistical analysis.

[15] A subsequent study (202) analyzed these data further. PDI results were for infants in the ≤1250 g birth weight subgroup.

[16] Prospective study.

[17] Maternal IQ was evaluated but not included in confounder analysis. The primary goal of the study was to examine effects of various concentrations of organochlorine compounds in cord serum.

[18] Part of the MRC National Survey of Health and Development; information on the duration of breastfeeding was obtained by interview.

[19] Tests at 26 and 53 y were not significant after adjustment for cognitive ability at 15 y.

[20] Only tests measuring visual-motor or visual-spatial outcomes were positive after adjustment.

LCPUFA supplementation on performance in cognitive or behavioral tests, and, as indicated in the Introduction, on visual acuity. The 2 Cochrane reviews (73, 74) pointed to 1 positive problem-solving study in term infants (205) and to 2 studies in preterm infants (206, 207) that reported shorter look durations, which are considered to indicate greater development of attentional processing. However, the Cochrane reviewers concluded overall that available evidence did not support suggestions that LCPUFA supplementation benefited neural development. Other reviewers acknowledged that results in RCTs were mixed, particularly in term infant studies (57, 72, 79) but also pointed out that tests measuring visual attention (206-208) provided consistently positive results in human RCTs in both preterm and term infants, which paralleled similar results in nonhuman primate studies (136).

As shown in Table 1, only 3 of the RCTs listed that compared LCPUFA-supplemented and -unsupplemented formula-fed children were not discussed in previous reviews. Auestad et al (163), in a 39-mo follow-up study of term infants, did not observe a significant difference in performance on several mental and motor development tests (or in visual acuity). Bouwstra et al (164), also in a study of term infants, reported positive effects ($P < 0.05$) of LCPUFA supplementation at 3 mo of age by using a test that assessed the quality of general movements considered to be an indicator of brain function. And Fewtrell et al (184) reported weakly significant ($P = 0.04$) enhancement effects in 18-mo-old children fed fish oil–supplemented formula as preterm infants in the Mental Development Index component of the Bayley Scales.

Other experimental designs

The study of Colombo et al (185) listed in Table 1 adds to the series of positive results obtained in look duration tests. This study reported a positive correlation between maternal erythrocyte phospholipid DHA at birth and performance in look duration tests over the first 18 mo of life. Other studies listed in Table 1 that were not previously discussed by reviewers include a negative study that examined possible correlations between performance in several mental development tests at 4 and 7 y of age and LCPUFA blood concentrations at birth or at the time of testing (179, 180), a study reporting a positive correlation between maternal plasma phospholipid DHA concentrations and the maturity of sleep patterning in infants (186), and a study, some results of which were significant, that compared performance in several mental and motor development tests of ≈1-y-old term infants fed infant formulas differing in ALA content (187).

STUDIES IN ANIMALS

The great strength of animal studies is that they afford the opportunity for more flexibility in design and in the ability to control experimental variables than can be achieved in human studies. The most commonly used experimental design in animals involves limiting the dietary supply of ALA, the precursor of DHA, which is not feasible in human studies because ALA is an EFA. Previous reviewers of animal studies (49, 57, 119, 146, 147) concluded that reproducible effects of dietary restriction of ALA on look duration in nonhuman primates suggested possible involvement of DHA in cortical pathways associated with visual attention (57) and also concluded that effects on cognition in rodent studies were shown in tests that involved sensory pathways other than the visual (31, 49, 209-211).

Most animal studies have been conducted by using rodents. Although a detailed comprehensive methodologic review in 1992 (146) discussed early rodent studies, and more recent reviews have critically discussed some subsequent work (49, 57, 119, 147), there is now quite a large database of rodent behavioral studies that have not been critically reviewed. More than 30 rodent studies (102–105, 148, 212–238) have been published since the 1992 comprehensive methodologic review of Wainwright (146). More than 20 of these reports were published since 1999 (**Table 2**). (Investigations that focused solely on visual function are not included here.) The results reported in these studies are briefly summarized below.

First, almost all of the 20 rodent studies listed in Table 2 reported significant deficits in test performance by subjects compared with controls on at least some tasks in at least some n−3–restricted groups. These results support earlier studies, previously reviewed (49, 57, 119, 146, 147), that suggested an association between a diet severely restricted in n−3 FAs during development and poorer performance of offspring in tests designed to measure cognitive or behavioral ability. This important basic conclusion is tempered by the fact that performance differences are typically not large, are observed on some tasks and not others, often are not observed across multiple test sessions, and have not been observed in all reports (further discussed below).

Second, performance deficits were reported in a variety of tests, which suggests that the effect on performance is not test-specific. For example, among studies listed in Table 2, tests for which effects were reported included the Morris water maze (105, 212, 215-217, 219, 220, 232), 8-arm radial maze (229, 230), elevated plus maze (220, 234), active avoidance (227, 235), olfactory-cued (214, 216), and brightness discrimination (224, 227) tests.

Third, 8 of the studies in Table 2 supplemented n−3–restricted animals with DHA, DHA+AA, DHA-rich oils, or DHA and additional n−6 FAs and compared the animals' performance with that of n−3–restricted controls (40, 212, 217, 224, 229, 230, 234, 235). All of these studies reported that performance was significantly enhanced in the supplemented groups.

Fourth, all but 5 studies (218, 219, 233-235) listed in Table 2 also measured brain concentrations of LCPUFAs. In almost all cases, brain DHA concentrations were significantly lower in n−3–restricted groups than in controls, and several investigations pointed to the importance of AA as well (224, 227, 228). In addition to conducting performance tests and determining brain concentrations of LCPUFAs, several studies recorded changes in biochemical indicators of brain function in n−3–restricted and control groups (226, 229, 235).

Fifth, limited evidence of a dose-response effect is reported in 2 studies (215, 240). We also note several reports in which performance deficits were observed in some groups by using dietary protocols that resulted in significantly less reduction in brain DHA than is seen in most multigeneration ALA-restriction studies (38, 40, 105, 148).

Factors involved in assessing and interpreting studies in rodents

An in-depth methodologic review of all of the studies discussed above is beyond the scope of this review. We note, however, that a thorough critical evaluation of results should take into account a number of methodologic and other issues, many of which have been discussed by previous reviewers (49, 51, 52, 55, 57, 118, 119, 146, 147, 209, 241, 242). Several of these issues are briefly summarized below.

Potential confounders not usually evaluated cannot be excluded as alternative explanations for results in many experiments, as is discussed in several reviews (4, 57, 119, 243, 244). Most tests measure a combination of performance and behavioral characteristics in addition to cognition per se; Wainwright (119) and Wainwright et al (148) provide further discussion of this topic. For example, in addition to learning ability, commonly used water and radial maze tests involve locomotor ability and visual recognition of cues, and brightness discrimination tests require visual ability. As discussed above, chronic dietary restriction of ALA adversely affects the development of visual function, which must be considered to potentially influence the results of these tests. Other potential confounders are long-term severe imbalance in the ratio of n−6 to n−3 FAs or other effects of chronic ALA restriction. In addition to its role as precursor of DHA, ALA is an important source of energy, is a precursor for the synthesis of saturated and monounsaturated FAs (51, 245)—that comprise some 75% (by wt) of the FAs present in brain tissue (4), including myelin (246)—and is believed to be required for the development of an adequate arterial supply (245, 247). In addition, the very high n−6:n−3 used to generate n−3–deficient groups in the rat experiments may result in an unnaturally high level of inflammatory activity (248, 249), which could affect the general health or vigor of the animals and thus influence their ability to perform in cognitive and behavioral tests.

TABLE 2
Animal studies (1999–2004) that tested effects of diets differing in n−3 fatty acid content during development on behavior[1]

Reference	Protocol	Performance tests
Rodents		
Carrie et al (217, 219, 220); Frances et al (218)	Swiss OF-1 F1 mice (most studies); 3 basic designs: *1*) comparison of n−3–deficient animals (ratio of n−6 to n−3 >1200 compared with 6) (218, 220); *2*) supplementation protocol (219); and *3*) comparison of n−3–deficient animals supplemented with DHA at different ages (217)	Reward test aimed at determining the interest level of animals in responding to a pleasurable stimulus (218); Morris water maze (217, 219, 220); elevated plus maze test (220)
Gamoh et al (229, 230)	Wistar F2 rats; fish oil–deficient diets and then DHA administered intragastrically 5 wk before testing	8-Arm radial maze test at 10 wk (230) or 100 wk (229) of age
Ikemoto et al (224)	Donryu F1 rats; semipurified diets supplemented with safflower or perilla oil	Brightness discrimination learning test at 11 wk of age
Levant et al (40)	Long-Evans F1 rats; n−3–deficient diets from conception to weaning	Tests for haloperidol-induced catalepsy, locomotor activity, and withdrawal latency to a thermal stimulus at ≈2 mo
Moriguchi et al (212, 215); Greiner et al (214, 216); Catalan et al (213)	Long-Evans F1 or F2 rats; n−6:n−3, ≈350 and 5	Morris water maze or an olfactory-cued discrimination test conducted in animals ranging in age in various studies from 8 to 13 wk
Takeuchi et al (234, 235)	F1 Wistar rats; diets lacking n−3 FAs or with n−6: n−3 of 0.39; at 3 wk of age, DHA was administered to one group	Active avoidance and 3-panel runway test (235) or plus maze test (234) at 8 wk of age
Umezawa et al (227)	Senescence-resistant F1 mouse strains; supplementation with safflower or perilla oil	Active avoidance (Sidman) and brightness discrimination learning tests at 15 mo of age
Wainwright et al (105, 231, 233); Clements et al (232)	Long-Evans rat pups artificially reared by gastrostomy with rat milk substitutes variously supplemented with DHA or AA or both (105); F1 B6D2F1 mice; DHA in the brain manipulated by changing the concentration of γ-linolenic acid (an n−6 FA) in the diet (231); adequate diets of two rat strains—spontaneously hypertensive rats and their progenitor strain (Wistar-Kyoto)—were supplemented with AA and DHA beginning at weaning (232); F1 B6D2F1 mice; diet with a very low n−6:n−3 (n−6 was linoleic acid; n−3 was DHA) (233)	Morris water maze (105); behavioral development tested at 12 d of age, and adults tested in elevated plus maze (231); a version of the Morris water maze at 8 wk of age (232); a behavioral test battery at 32 d of age (233)
Piglets		
Ng and Innis (38)	Piglets fed low-PUFA or high-PUFA diets (+DHA and AA) starting at 1 d of age	Elevated plus maze at 18–22 d of age
Monkeys		
Champoux et al (239)	Rhesus macaque infants were fed standard formula or standard formula supplemented with DHA and AA	Neurobehavioral assessments at 7, 14, 21, and 30 d of age

[1] F1, first generation offspring of treated F0 females; F2, second-generation offspring; DHA, docosahexaenoic acid; FAs, fatty acids; AA, arachidonic acid; PUFA, polyunsaturated fatty acid.

Statistical considerations not taken into account can affect conclusions about the positivity (or negativity) of experiments, as well as the interpretation of positive results. Wainwright (250) has discussed several design and analysis issues with respect to multigenerational studies in multiparious species, and she emphasizes that weak or marginal results must be viewed with caution. With reference to the studies included in this review, we point particularly to 3 important statistical considerations. First, as Wainwright discussed (250), the inflation of sample size by using the number of pups instead of the number of litters as the unit of measure results in an increased potential for false positives unless appropriate cluster design adjustments are made. Second, in correlational analyses, if group means for the measures being correlated are significantly different, a simple correlation across groups does not necessarily indicate a correlation for individual animals. Third, because "learning" implies a change over time, it is inappropriate to conclude that there is a learning difference between compared groups unless the statistical analysis indicates a group × time interaction.

As discussed by many reviewers (4, 119, 147), test results obtained in experiments involving severe depletion of brain DHA have uncertain relevance to effects that might be expected under less severe conditions. In nonhuman primates (64) and piglets (38), DHA concentrations in the brain are dramatically reduced after the maternal dietary supply of ALA is limited during one gestational period. In rodents, a longer period of chronic ALA restriction is required to achieve a comparable reduction because of athe more efficient biosynthesis of DHA from ALA in rodents (251). Thus, most rodent studies have been conducted with animals whose mothers or even mothers and grandmothers were raised on diets limited in ALA. In animals whose supply of DHA has been limited in this way, during the perinatal period (when rapid accumulation of

brain DHA would normally occur), the place of DHA is taken primarily by another FA, docosapentaenoic acid (DPA; 22:5n−6), which differs from DHA in just one double bond—the n−3 double bond (101, 252).

Performance of n−3–restricted rats and mice in the Morris water maze

The Morris water maze is the most commonly used learning and memory test in the rodent studies reviewed (102–105, 148, 212, 215-217, 219, 220, 223, 232, 236). Below, we briefly discuss the subset of these studies that included a determination of brain concentrations of DHA and compared an n−3–restricted group to "n−3–adequate" controls. It is noted that the 10 studies selected (103–105, 148, 212, 215-217, 220, 223) included additional outcome measures or other comparisons not discussed in this example. Some of these studies have been reviewed elsewhere (49, 119, 241).

In rats, one laboratory reported reproducibly better performance of controls than of severely n−3–restricted animals in the "place" version of the test (212, 215, 216). Using the same version of the test, a different laboratory reported a nonsignificant trend ($P > 0.08$) in one study (148) and a negative result for a "saturated fat" group in a larger study (105). The latter 2 studies also used the "delayed-matching-to-place" or "working memory" version of the test, and both reported significant positive results [a diet × trials interaction, $P < 0.01$ (148) or $P < 0.05$ in a planned comparison (105)].

In mice, a positive result ($P < 0.05$) was reported for a saturated fat group, but not for an n−3–restricted group in the same study (103). Positive results were also reported in 2 other studies (217, 223). However, in one of those studies (217), significance relied on a post hoc comparison of results in a single session conducted on a data set after a nonsignificant ANOVA, and, in the other study (223), significance was marginal ($P = 0.044$) and relied on the use of a one-tailed test. Two other mouse studies reported negative results (104, 220).

Although rats are known to perform better than mice in the water maze (253–255), it may be of interest that, among the studies discussed above, positive results were obtained in both species in the place version of the test when reductions in brain concentrations of DHA in the restricted group exceeded 80% (103, 212, 215, 216). Results were negative or marginal when brain reductions were less, ie, 25% (217), 41% (220), 50% (103), 60% (223), and 53% (104). It may also be of interest that positive results were obtained in rats with less severe depletion of brain DHA, ie, 38% (105) and 51% (148), in the delayed-matching-to-place version of the water maze, which, as discussed previously (148), may be more sensitive for detection of effects of n−3 restriction than is the place version.

Docosahexaenoic acid supplementation studies

Only a few studies in animals have examined effects of LCPUFA supplementation of normal diets during development on performance in cognitive or behavioral tests. Among the rodent studies listed in Table 2, only one (105) used a study design analogous to that of formula comparison studies in humans. That study did not find that supplementation with DHA or AA (or both) improved subsequent performance on 2 versions of the Morris water maze, despite the fact that feeding diets with normal concentrations of EFAs supplemented with different amounts of AA and DHA to artificially reared pups was found to result in a range of different concentrations of AA and DHA in forebrain phospholipids (256). One other study listed in Table 2 included dietary groups for which n−6:n−3 was within the range considered roughly normal (ie, ≈5–10) or enriched for n−3 FAs (219). That study reported some changes in performance on the Morris water maze in animals fed the n−3–enriched diet but also reported adverse effects in older animals fed the n−3–enriched diet for extended periods. A study in rhesus macaque infants (239) reported that those fed LCPUFA-supplemented formula had stronger orienting and motor skills than did those fed standard formula.

SUMMARY AND CONCLUSIONS

Each of the 3 major experimental designs reviewed contributes some evidence that is relevant to a discussion of possible causal linkages between altered brain concentrations of DHA during the perinatal period and subsequent cognitive or behavioral performance. (As indicated above, effects of DHA availability during development on visual function are not a subject of this review.) Evidence from chronic dietary restriction rodent studies that is most relevant to the issue of causality shows that the addition of DHA to diets of animals whose brain concentrations of DHA have been severely reduced restored control performance levels (40, 212, 217, 224, 229, 230, 234, 235). As discussed in this review, the relevance of these results to effects that might be expected in humans under less severe conditions is uncertain. Formula comparison and maternal supplementation studies in humans and ALA dietary restriction studies in nonhuman primates both link the availability of n−3 LCPUFAs to the development of visual attention (136, 185, 206-208), although, as discussed, it is difficult to exclude as an alternative explanation for these results the possible confounding due to effects of DHA on visual function. We also point to formula supplementation (164, 239) or maternal plasma correlational (186) studies in humans or monkeys that suggest enhanced neuromotor development in infants with higher DHA status. Positive results in breastfeeding studies are seriously eroded by adjustment for multiple covariates, and residual positive effects cannot be attributed to LCPUFAs because of other potentially active constituents in breast milk. Nevertheless, it is of interest that these studies consistently showed a positive association between breastfeeding and performance across a wide range of different tests, even after adjustment for at least some potential confounders.

Clearly, the experiments most capable of providing definitive evidence that is directly relevant to human exposure conditions are RCTs in humans. As reviewed above, these trials have often not shown an effect of LCPUFA supplementation on cognitive or behavioral performance, and some reviewers have considered that, overall, the evidence was insufficient to conclude that LCPUFA supplementation benefited development (73, 74). Understanding why most RCTs have yielded mixed results, particularly in light of more consistently positive effects observed in rodent tests and human breastfeeding studies, is of obvious importance and has been discussed by a number of reviewers and investigators (4, 49, 77, 79, 122). The suggestion of previous reviewers (57, 124) that differences in the sensitivity of global tests, such as the Bayley Scales, and of tests targeted at more

specific neural domains, such as look duration or problem-solving tests, might account for mixed results in RCTs was discussed earlier in this review. Other possible explanations include inadequate supplementation of DHA in formulas (49, 257), poor study quality (77), the ability of term infants fed unsupplemented formulas to synthesize their own DHA (73), an absence of cognitive deficits when differences in brain concentrations of DHA are small due to brain plasticity (ie, the ability of the brain to adapt), or the inability of performance tests to detect subtle differences in performance that result from relatively small differences in brain concentrations of DHA (105, 210).

The relative merits of hypotheses that invoke brain plasticity or difficult-to-detect weak effects due to small decreases in brain DHA are important to follow up. The former hypothesis suggests that there may be no adverse consequences of relatively small reductions in brain DHA, whereas the latter suggests that relatively small reductions could result in subtle performance deficits that are difficult to detect. If experts can agree on a reliable marker, constructing a dose-response curve that relates the degree of reduction in brain DHA to effects on neural function would be of great value. It appears to us from our reading of the literature that cognitive and behavioral tests may not be sensitive or consistent enough to permit such an analysis, but some biochemical markers, such as dopamine inducibility (36, 38), may be useful.

Most of the explanations discussed above focus on the possibility that negative results in RCTs may be false-negative. Following some discussion of this point in the literature (146), we note that it is equally important to consider the possibility that positive results are false positives. In addition to the need to better identify confounders and methodologic sources of possible bias, as discussed, we note the value of independent replication to confirm positive results.

It is of interest to examine the degree to which evidence from the 3 major experimental systems reviewed satisfies conditions of causality. Causal criteria [slightly adapted from the original formulation (258)] require evidence of *1*) a consistent association, *2*) a plausible biological rationale, *3*) an ability to experimentally manipulate the effect, *4*) a dose-response relation, and *5*) specificity of cause and effect. Whereas some of these 5 criteria are satisfied by some experimental results reviewed, others are not. First, as detailed above in this review, all 3 major experimental systems reviewed (ie, human breastfeeding studies, human formula comparison RCTs, and animal ALA dietary restriction studies) show consistent associations under certain conditions. Second, mechanistic studies are not discussed in this review, but plausible biological rationales have been suggested, although not proven; recent references and reviews are cited in the Introduction. Third, experiments in rodents that restored performance by supplementing severely ALA-restricted animals with DHA provided some evidence of ability to manipulate the effect. Fourth, as indicated above in this review, limited evidence suggesting a possible dose-response effect was provided in 2 rodent studies (215, 240) and in a study that correlated concentrations of DHA in the blood of breastfed infants with enhanced performance (188). The only studies reviewed that provided consistent evidence of a dose-response effect are breastfeeding studies that correlate duration of breastfeeding with improvements in test performance (*see* Table 1). Fifth and finally, as discussed above, the interpretation of results in all 3 experimental designs is complicated by uncertainty as to the specificity of cause and effect. With respect to "cause," DHA is not specifically pointed to in breastfeeding studies because there are numerous factors in breast milk besides DHA that could affect the outcome. DHA is also not specifically implicated in ALA-restriction studies in animals because of FA imbalance or other possible effects of severe and chronic ALA restriction. Human formula–comparison studies are the least uncertain with regard to the specificity criterion. However, most of these studies do not supplement formulas only with DHA. With respect to "effect," possible effects of DHA on endpoints other than cognition or behavior, such as visual function, could bias the results of numerous tests, as indicated above. Thus, on the basis of these 5 causal criteria, the experimental results reviewed here supply some evidence that is not inconsistent with a causal connection between DHA availability and cognitive function, but they do not show causality.

Finally, we comment briefly on our view of the relevance of results reviewed here to the question of whether infant formula should be supplemented with DHA. The experiments most directly relevant to this question obviously are human RCTs that compare performance in children fed LCPUFA-supplemented or -unsupplemented formulas. As discussed, results from these RCTs are mixed and have not consistently shown a positive effect of supplementation on cognitive or behavioral function other than intriguing results from a relatively small number of studies. Results of human breastfeeding studies, though seriously confounded, are not inconsistent with a need for supplementation, but the studies do not provide direct or clear evidence. We consider that animal studies provide the most convincing and consistent evidence linking a decrease in brain concentrations of DHA to altered performance on cognitive or behavioral tests. However, effects are not large, despite the fact that the studies were conducted under severe dietary conditions, and results are difficult to extrapolate to the human situation. In our view, the main contribution of these animal studies to the discussion of infant formula supplementation is that they suggest the possibility, as discussed by others, eg, Wainwright (119), that small differences in brain concentrations of DHA, such as most likely occur between infants fed supplemented or unsupplemented formulas, may result in subtle effects that currently are difficult to detect but could be significant.

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